

Promoter competition as a mechanism of transcriptional interference mediated by retrotransposons

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Enhancers can function over great distances and interact with almost any kind of promoter, but insulators or promoter competition generally limit their effect to a single gene. We provide *in vivo* evidence that retroelements may establish promoter competition with their neighboring genes and restrict the range of action of an enhancer. We report that the retroelement *Idefix* from *Drosophila melanogaster* inhibits *white* gene expression in testes by a promoter competition mechanism that does not occur in the eyes. The sequence specificity of the two TATA-less promoters of *white* and *Idefix* is a prime determinant in the competition that takes place in tissues where both are transcriptionally active. This study brings to light a novel mechanism whereby transcriptional interference by an active retrotransposon may perturb expression of neighboring genes. This capacity to interfere with the transcriptional regulation of their host, together with the facts that retroelements preferentially move within the germline and do not excise to replicate, suggest that these elements are *cis*-regulatory sequences able to imprint specific and heritable controls essential for eukaryotic gene regulation.

Keywords: *Drosophila/Idefix*/promoter competition/retrotransposons/transcriptional interference

Introduction

In eukaryotes, the tissue and spatial specificity of gene expression is regulated by interactions of proteins with short DNA sequences called enhancers. Enhancers exert long-distance effects independently of their position and orientation. Moreover, enhancers have been shown to be able to drive the transcription of a heterologous gene if located in its vicinity. This raises the question of how an enhancer specifically activates its target gene without affecting adjacent genes. Two main mechanisms have been described for regulating the interaction of a shared enhancer with a specific target promoter: insulator DNAs and promoter competition.

In the first mechanism, chromosomes are divided into distinct domains of gene action by insulator sequences: a given enhancer will then interact with a defined promoter only if both reside within the same domain. The elements *scs* and *scs'* identified in the flanking regions of the

Drosophila hsp70 locus were the first insulators to be identified (Udvardy *et al.*, 1985; Kellum and Schedl, 1991, 1992). Additional insulator elements have since been identified, including the suppressor of *Hairy wing* insulator within the *gypsy* retroelement, the Fab-7 and Fab-8 insulators within the *bithorax* complex of *Drosophila* (Geyer and Corces, 1992; Hagstrom *et al.*, 1996; Zhou *et al.*, 1996, 1999; Barges *et al.*, 2000) or, in vertebrates, the insulator present at the chicken β -globin locus (Chung *et al.*, 1993, 1997). All the insulators described so far block gene expression only when located between a distal enhancer and a target promoter. Recently, a *cis*-regulatory element that permits an enhancer to bypass an insulator has been identified. This element, called promoter targeting sequence or PTS, allows distal enhancers to overcome the blocking activities of insulators (Zhou and Levine, 1999).

According to the promoter competition model, the inherent properties of promoters and enhancers allow only certain combinations to interact, while other combinations are inefficient. Consequently, a shared enhancer can activate multiple genes, but selects the promoter region of a single one. Activation of the preferred gene precludes expression of the neighboring genes. Promoter competition was first identified in the chicken globin gene cluster (Choi and Engel, 1988; Foley and Engel, 1992) and has since been implicated in the regulation of genes within the ANT-C in *Drosophila* (Ohtsuki *et al.*, 1998) and in the regulation of mammalian Hox genes (Herauld *et al.*, 1997; Sharpe *et al.*, 1998). Promoter competition is likely to be mediated by the promoter specificity. Three different core promoter elements located within a 50–60 bp sequence flanking the transcription start site have been identified: the TATA box, the initiator element (Inr) and the downstream promoter element (DPE) (Burke and Kadonaga, 1996). Promoters are subdivided into two classes: (i) TATA-less promoters, which contain conserved copies of Inr and/or DPE; and (ii) TATA promoters, which do not require Inr or DPE elements to initiate transcription (Burke and Kadonaga, 1996). It has been shown that some enhancers preferentially activate TATA promoters when given a choice between a linked TATA and a TATA-less promoter. In the ANT-C complex, the enhancer AE1 preferentially activates the TATA promoter of the *fushi tarazu* (*ftz*) gene over the TATA-less promoter of *Sex combs reduced* (*Scr*) (Ohtsuki *et al.*, 1998). In contrast, some enhancers interact preferentially with DPE-containing promoters over promoters with a TATA box (Butler and Kadonaga, 2001). These findings suggest that proteins bound at the enhancer and promoter regulatory elements are probably implicated in the promoter competition mechanism.

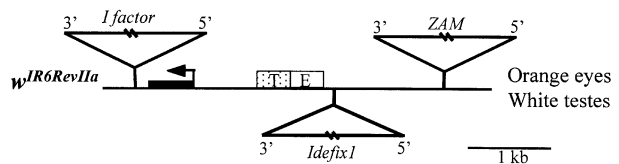
The arrival of additional enhancers and promoters due to recent insertions of transposable elements at a defined

locus can affect the relationship established between the promoter of a nearby gene and its enhancers. Such alleles can thus be powerful tools to help understand the mechanisms by which enhancers activate promoters. We made use of recurrent insertions affecting the *white* locus of the *Drosophila* genome to examine the modifications of the enhancer–promoter interactions established after such insertions. Four alleles of the *white* gene from successive insertions of three transposable elements have already been described (Desset *et al.*, 1999; Conte *et al.*, 2000). The w^{IR6} allele is due to the insertion of an *I factor* within the first intron of the *white* gene. This insertion is responsible for an alteration of *white* gene splicing and thus a decrease in the wild-type transcripts of the gene, which leads to an orange instead of brick-red eye phenotype (Lajoinie *et al.*, 1995). The $w^{IR6RevI}$ allele identified in a line referred to as RevI results from the insertion of the retroviral element ZAM 3 kb upstream from the w^{IR6} transcription start site (Leblanc *et al.*, 1997). These flies display brick-red eyes. Spontaneous mutants with orange eyes among the red-eyed population were recovered in the RevI line and established as lines denoted RevII. These mutations arise from an insertion of the retroviral element, denoted *Idefix*, 1.7 kb upstream of the *white* gene promoter, leading to the $w^{IR6RevII}$ alleles (Figure 1A). Finally, an allele called $w^{IR6RevIV}$, identified in a line denoted RevIV, derives from $w^{IR6RevII}$ through an additional insertion of *Idefix* at the 3' end of the first *Idefix* and in the opposite orientation. Flies carrying this *white* allele display a full reversion of the $w^{IR6RevII}$ eye phenotype to brick-red (Desset *et al.*, 1999).

In a previous paper, we examined the molecular mechanisms by which ZAM and *Idefix* modulate the expression of the *white* gene in the eyes of the RevI, RevII and RevIV lines. We reported that ZAM contains an enhancer in its 5'-untranslated region (UTR) capable of activating downstream *white* gene expression in the eyes, while *Idefix* carries an insulator able to block the activation by the ZAM enhancer in this tissue (Conte *et al.*, 2002; Figure 5A and B).

Since the *white* gene is controlled by two enhancers, one driving its expression in the eyes and the other in the testes, lending them a yellow color, we focused our attention on the testes color of these different lines. Here, we report that all the lines w^{IR6} , RevI and RevIV display yellow testes, while the RevII lines, whose structure is shown in Figure 1A, display white testes. By transgenic experiments, we show that the upstream transcriptionally active *Idefix* is able to repress the downstream *white* gene, a phenomenon known as 'transcriptional interference'. Our data indicate that in RevII, the promoter of *Idefix* is able to compete with the *white* gene promoter for the testes enhancer. This competition is orientation dependent, since an isolated novel RevII line with an *Idefix* insertion in the opposite orientation displays yellow testes. These results bring to light a novel mechanism by which transposable elements can interfere with the transcriptional regulation of their host, i.e. promoter competition. We report that this competition occurs between two TATA-less promoters that possess distinct properties enabling them to be selected or not by *cis*-regulatory sequences and establish promoter competition.

A



B

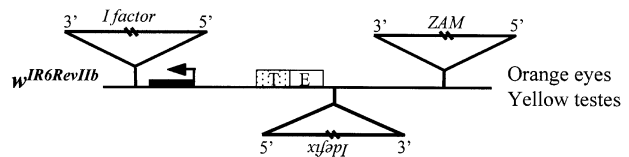


Fig. 1. Molecular structures of the $w^{IR6RevII}$ alleles and associated eye and testis phenotypes. The structure of the *white* alleles is represented as follows: the *white* gene transcription unit is indicated by a line, its first exon by a black box, and its transcription start site by an arrow. DNA upstream of the gene is shown as a line, and the *white* enhancers (E for eyes and T for testes) as boxes. The insertion sites of the *I factor*, ZAM and *Idefix* are indicated by triangles. The names of the *white* alleles are shown on the left, and the eye and testes colors of the flies bearing these alleles are indicated on the right.

Results

Idefix interferes with *white* gene expression in testes in an orientation-dependent manner

The wild-type expression of *white* is driven by two enhancers located close together at 1.5 kb upstream of the *white* gene promoter (Figure 1). In a wild-type context, the testes enhancer leads to a yellow phenotype of the testes. When this enhancer is not functional, no coloration is observed and the testes are white. Focusing our attention on the testes color of the RevI, RevII and RevIV lines, we found that, as for w^{IR6} , RevI and RevIV displayed a yellow testes phenotype, compared with a white one in RevII.

The yellow pigmentation of the RevI line potentially could be due to the activity of both the endogenous testes enhancer of *white* and an enhancer provided by the insertion of ZAM. The ZAM sequence is composed of two long terminal repeats (LTRs) flanking a long 5'-UTR and three open reading frames (ORFs) (Leblanc *et al.*, 1997). Since the 5'-UTR and LTRs of diverse retroviral sequences have been found to be responsible for the deregulation of genes located close to their insertion, we tested the potential involvement of the 5'-UTR and the LTR of ZAM in their effects on the *white* gene. We analyzed the testes color of initially established transgenic lines to clarify the influence of ZAM on the eye color of the RevI line (Conte *et al.*, 2002). Constructs called pUzW or pLzW, in which the 5'-UTR or the LTR of ZAM have been inserted upstream of the mini-*white* gene, respectively, were injected in the w^{1118} line. The latter displays a mutation of *white* leading to white eyes and testes. Among five independent transgenic lines analyzed for each of the transgenes, none displayed yellow testes (data not shown).

This result indicates that *ZAM* does not act as an enhancer of the *white* gene that is able to direct its expression in the testes. The yellow phenotype of the RevI testes must therefore derive solely from activation of the *white* gene by its own testes enhancer.

Analysis of the RevII lines revealed that the relationship established between the promoter of *white* and its testes enhancer was modified upon the insertion of *Idefix* 1.7 kb upstream of the *white* transcription start site. Testes are white in these lines instead of yellow. Two major observations prompted us to search for the molecular mechanisms responsible for this variation of *white* gene expression in RevII testes. First, the ability of *Idefix* to act as an insulator, described by Conte *et al.* (2002), could not account for the white phenotype of these testes, since *Idefix* is not positioned between the testes enhancer and the *white* promoter, but upstream of both (see the structure of the $w^{IR6RevII}$ allele, Figure 1A). Secondly, we recently established two new RevII lines from the RevI stock. The structure of their $w^{IR6RevII}$ allele is similar to that described in Desset *et al.* (1999) and in Figure 1A, but with one main difference: the *Idefix* insertion is in the opposite orientation. This novel allele is schematized in Figure 1B. Hence, two categories of RevII lines exist that differ in their *Idefix* orientation. Although both display an orange eye phenotype readily explained by the presence of the insulator able to counteract the enhancer effect of *ZAM*, they display, surprisingly, different testes colors. As reported above, the first RevII lines identified displayed white testes, while the last ones displayed yellow testes. These will be identified subsequently as $w^{IR6RevIIa}$ alleles in RevIIa lines when *Idefix* is inserted in the 5'–3' orientation with regard to the *white* gene transcription start site or as $w^{IR6RevIIb}$ alleles in RevIIb lines when *Idefix* is inserted in the 3'–5' orientation (Figure 1A and B, respectively). Thirdly, when two *Idefix* are present upstream of *white* and in the opposite orientation, as in RevIV, the testes are then yellow. These findings suggest that the molecular mechanism responsible for the mutagenic effect of the element present in RevIIa can be suppressed by an additional element in the other orientation.

From these observations, we conclude that the enhancer–promoter dialog established between the *white* gene and its testes enhancers may be modified upon arrival of *Idefix*. This modification is independent of the insulator function of *Idefix*. It depends on the orientation of *Idefix* and can be suppressed by an additional *Idefix* if inserted in the opposite orientation.

The LTR of *Idefix* is able to break the dialog between the testis enhancer and the *white* gene promoter

To elucidate the molecular mechanisms responsible for this variation in the testes color between ReIIa and b lines, we constructed transgenes designed to mimic the structure of these alleles and analyzed their expression in transgenic flies.

Idefix is composed of two LTRs flanking a 5'-UTR and three ORFs (Desset *et al.*, 1999). Plasmids called pLiEwW were constructed to test the impact of the LTR of *Idefix* on *white* gene expression. They display the LTR (Li) placed upstream of the *cis*-regulatory sequences of *white* (Ew) and

the mini-*white* gene (W). Additionally, the LTR is flanked by FRT elements, which are targets for the *flp* recombinase action (Figure 2A and B). We reasoned that if the LTR of *Idefix* is responsible for the phenotype of the RevII testes, then it should also interact in the dialog between the *white* enhancers (Ew) and the downstream mini-*white* gene (W). These artificial constructs injected into flies carrying the w^{1118} null mutation should lead to transgenic flies with white or yellow testes depending on *Idefix* orientation in the transgenes. Transgenic flies bearing pLiEwW constructs were identified by their eye color due to the expression of the mini-*white* gene in this tissue and established as lines denoted pLiEwW from the name of the injected plasmid. These lines were then subjected to the *flp* recombinase action. To this end, they were crossed to flies expressing the *flp* recombinase under the control of a heat shock promoter. Expression of *flp* in the descendants results in recombination between the two FRTs and deletion of the intervening LTR of *Idefix*. This yielded flies denoted pLiEwW-*flp*. This strategy permits an assessment of the *white* gene activity in the presence (pLiEwW flies) and absence (pLiEwW-*flp* flies) of *Idefix* LTR to avoid any complications in interpretation due to position effects. In addition, PCR experiments were performed on DNA extracts from heat-shocked and non-heat-shocked flies to check the excision of the FRT-flanked fragment in all the subsequent experiments (data not shown).

Two series of pLiEwW transgenic flies were tested: (i) pLiEwWa flies bearing the LTR of *Idefix* in the 5'–3' orientation according to the mini-*white* gene expression, as in RevIIa (Figure 2A); and (ii) pLiEwWb transgenic flies bearing the LTR of *Idefix* in the opposite orientation, as in RevIIb (Figure 2B). At least five independent transgenic lines were tested for each series of experiments.

Transgenic lines with the pLiEwWa construct displayed white testes (Figure 2A). When the LTR was excised after the *flp* recombinase action, the pLiEwWa-*flp* flies displayed yellow testes (Figure 2A). Thus, in this configuration, which mimics the $w^{IR6RevIIa}$ structure, *Idefix* is indeed able to block the activation of *white* gene expression in testes, and this effect is due to the presence of its LTR. In contrast, the pLiEwWb lines, in which *Idefix* LTR is inserted upstream of *white* and its enhancers, but in the opposite orientation, as in the $w^{IR6RevIIb}$ allele, displayed yellow testes (Figure 2B). When the LTR was excised after the *flp* recombinase action, the pLiEwWb-*flp* flies displayed yellow testes (Figure 2B).

From these findings, we conclude that the LTR of *Idefix* is able to block the dialog established between the testis enhancer and its *white* target promoter when inserted upstream. In addition, this interference with the transcriptional regulation of *white* is orientation dependent and only occurs when the LTR is in the same orientation as *white* gene transcription.

Both *cis*-regulatory sequences of *white* driving its expression in testes and in the eyes are present in our constructs. Surprisingly, no pLiEwW lines tested displayed any modification of the eye color on removal of the *Idefix* LTR (Figure 2A and B). This result therefore indicates that the enhancer-blocking activity of the LTR of *Idefix* on downstream enhancers may be specific to some enhancers and ineffective on others.

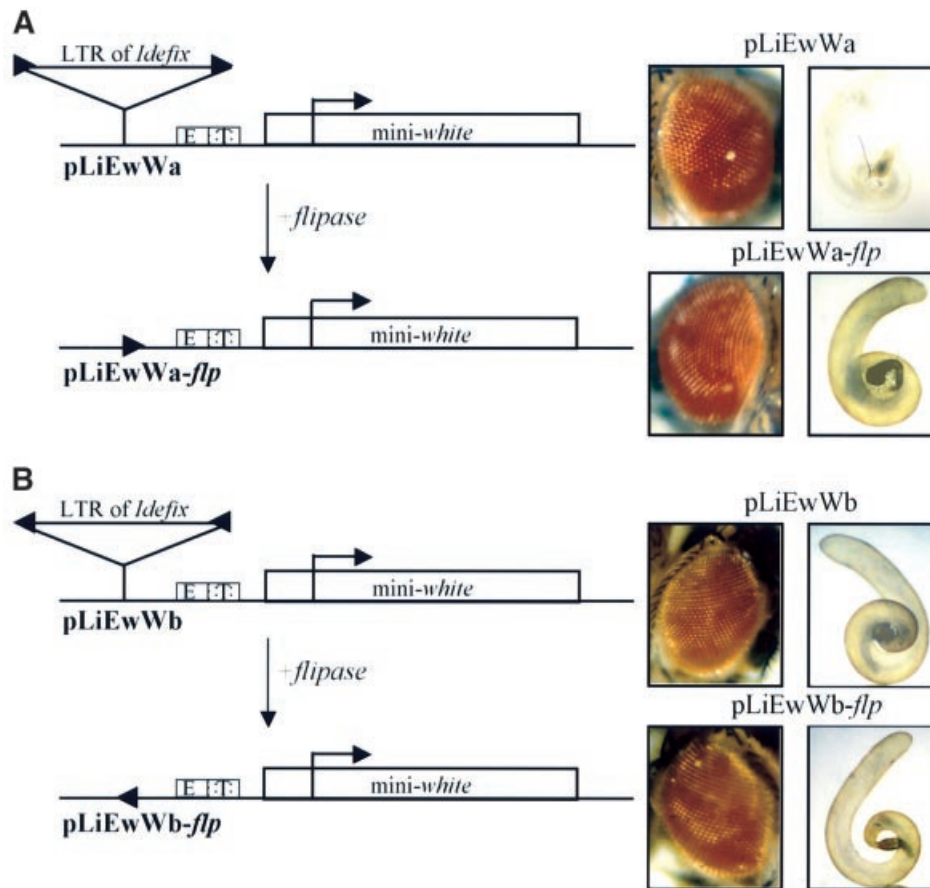


Fig. 2. The LTR of *Idefix* counteracts the activation effect of the downstream *white* testes enhancer. (A) The LTR of *Idefix* is inserted upstream of the *white* enhancers in the 5'–3' orientation and (B) in the 3'–5' orientation with regard to *mini-white* gene transcription. The structures of the *P* transformation vectors carried by the transgenic flies assayed for *Idefix* LTR inhibition effect are shown. The *mini-white* gene is represented by a white rectangle, its transcription start site by an arrowhead, and its eye and testes enhancers by boxes (E and T). The insertion site of the *Idefix* LTR upstream of the *white* enhancers is indicated by a triangle. The FRT sites, which flank the *Idefix* LTR, are shown by arrowheads. The names of the constructs are indicated below the figure, and the testes and eye colors of the flies bearing these constructs on the right. The second construct is the same as the first except that the LTR of *Idefix* has been removed via *flp*-mediated recombination.

The enhancer-blocking activity of the *Idefix* LTR is dependent on its transcription

Since the promoter of *Idefix* is contained within its LTR, we wondered whether the phenotypes observed in the transgenic lines were related or not to the transcriptional activity of *Idefix* within the transgenes. Previous experiments with a mutated LTR (*Li*⁴⁷⁷) displaying a point mutation in the Inr sequence of the *Idefix* promoter had shown that this LTR is unable to initiate transcription when fused to the *lacZ* reporter gene (see Materials and methods and Conte *et al.*, 2002). A series of experiments additional to those reported above was performed with the wild-type LTR of the constructs replaced by the mutated LTR. *Li*⁴⁷⁷ was inserted in the 5'–3' orientation upstream of the enhancers of *white* and the *mini-white* gene in constructs denoted pLi⁴⁷⁷EwW (Figure 4A). No modification of the eye color was observed before or after the removal of this LTR⁴⁷⁷ within the transgenic lines (data not shown). Additionally, the presence of this mutated LTR inserted upstream of *white* and its enhancers did not prevent the testis pigmentation, i.e. testes were yellow (Figure 4A). As expected, on removal of the *Idefix* sequence, pLi⁴⁷⁷EwW-*flp* lines also displayed yellow

testes due to the activation of *white* gene expression by its testes enhancer.

These results indicate that the *Idefix* LTR may exert an enhancer-blocking activity on specific downstream enhancers, but this interference with nearby genes depends on both its orientation at the locus and its transcriptional activity.

According to these results, the testis phenotype of the RevIIa flies (*w*^{IR6RevIIa} allele) is readily explained. In a 5'–3' orientation (according to the downstream *white* gene transcription), the *Idefix* promoter blocks interactions between the testes enhancer and the *white* gene promoter, resulting in a *white* testis phenotype. In contrast, *Idefix* inserted in the opposite orientation is not able to compete with the *white* gene promoter for its enhancer, and testes are yellow as observed in the RevIIb flies (*w*^{IR6RevIIb} allele).

Competition between *Idefix* and *white* promoters depends on their sequence

Two main mechanisms potentially can explain the transcriptional interference observed between *Idefix* and *white* in RevII lines: the readthrough transcription model or the promoter competition model.

In the readthrough transcription model, interference with *white* expression would result from readthrough transcription from the upstream *Idefix* promoter. In three series of RT-PCR experiments performed on the pLiEwWa lines, we searched for a chimeric transcript that would result from such a transcriptional interference and thus contain *Idefix* sequences plus *white* sequences. No chimeric transcript was ever detected in these lines.

In the promoter competition model, the inherent properties of the promoters and enhancers allow only certain combinations to interact, other combinations remaining inefficient. For example, it was shown that TATA

promoters are activated preferentially over TATA-less promoters by some enhancers (Ohtsuki *et al.*, 1998). In our system, the promoters of *white* and *Idefix* are both TATA-less promoters. Consensus sequences have been reported for Inr and DPE sequences that function co-operatively to direct accurate and efficient initiation of transcription in TATA-less promoters. The Inr and DPE motif of *white* and *Idefix* do not conform completely to the described consensus. The transcription start site of *white* conforms to the consensus of the Inr element (TCAGTT), which is sufficient to direct initiation in the absence of a TATA element. However, its DPE motif (GAAG) does not conform completely to the consensus G-T/A-C-G (Kutach and Kadonaga, 2000). For the *Idefix* transcription sites, its Inr (TCAGAG) at nucleotide 472 according to the *Idefix* sequence does not conform to the Inr consensus, while its DPE (GTCTG) at nucleotide 502 does (Tcheressiz *et al.*, 2002).

Thus we wondered whether the specificity of the *Idefix* promoter sequence was important for competition with the *white* gene promoter. To test this, transgenic experiments were conducted with a novel mutated LTR, called Li^{mut}. In this Li^{mut}, the Inr sequence of *Idefix* has been changed in such a way that a novel Inr sequence conforms to the consensus GCAGTT (Figure 3; Kutach and Kadonaga, 2000). We verified that this mutated LTR fused to a *lacZ* reporter gene is able to initiate *Idefix* transcription in testes like the wild-type LTR (see Figure 3, Li^{mut}LacZ compared with LiLacZ; Tcheressiz *et al.*, 2002). We then tested the ability of this LTR to compete with the *white* gene promoter. For this purpose, Li^{mut} was inserted in the 5'-3' orientation upstream of the endogenous enhancers of *white* and the mini-*white* gene in a construct called pLi^{mut}EwW

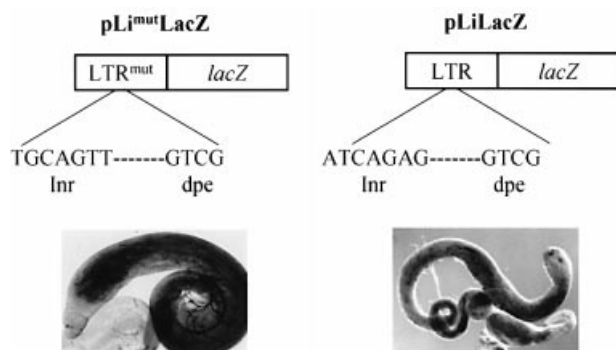


Fig. 3. The mutated LTR of *Idefix*, Li^{mut}, is able to initiate transcription. An Li^{mut} construct was subjected to *in vivo* transcription analysis by transgenic experiments. The name of the construct containing a Li^{mut}-*lacZ* fusion gene and its structure are indicated at the top. The *lacZ* reporter gene expression is detected in testes of flies bearing the Li^{mut}-*lacZ* construct. *LacZ* staining observed in a similar experiment with the wild-type LTR of *Idefix* (LiLacZ) is shown on the right (Tcheressiz *et al.*, 2002).

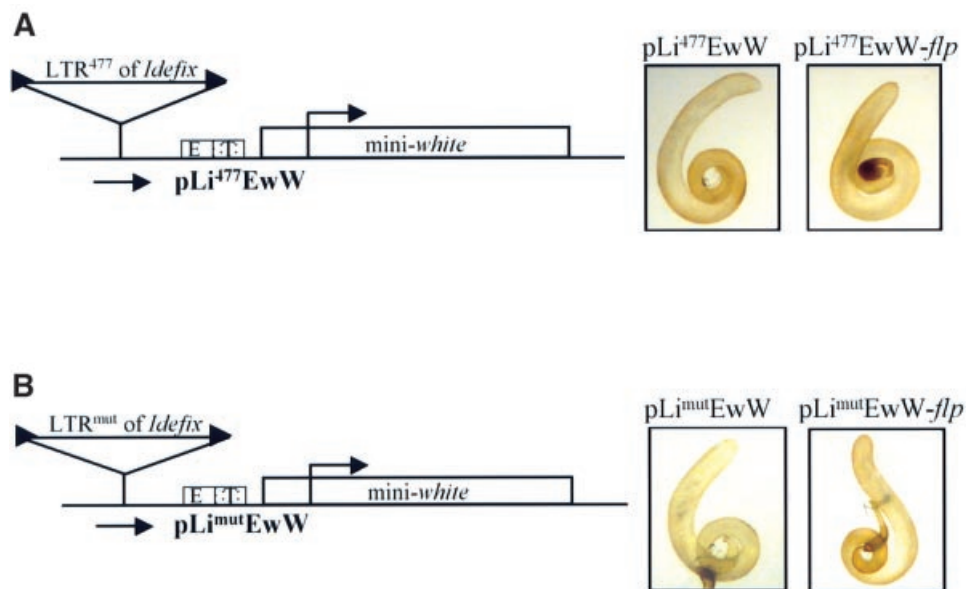


Fig. 4. Promoter competition between *white* and *Idefix* depends on *Idefix* promoter sequence. (A) The mutated LTR⁴⁷⁷ of *Idefix* (Li⁴⁷⁷) does not block the dialog between the *white* gene promoter and its testes enhancer when inserted upstream. Li⁴⁷⁷ is inserted in the 5'-3' orientation with regard to the mini-*white* gene orientation. This orientation and the corresponding name of the *P* transformation vector are indicated below the constructs. The testes color of the flies bearing the *P* transformation vectors is shown on the right before and after the *flp* recombinase action. (B) The mutated LTR^{mut} of *Idefix* (Li^{mut}) does not inhibit *white* gene expression driven by its testes enhancer, although it initiates transcription. The *P* transformation vector assayed for the effect of Li^{mut} on *white* gene expression is the same as in (A) except that Li⁴⁷⁷ of *Idefix* has been replaced by Li^{mut}. The orientation of Li^{mut} is indicated by an arrowhead below the construct. The testes color of the flies bearing the *P* transformation vectors is shown on the right before and after the *flp* recombinase action.

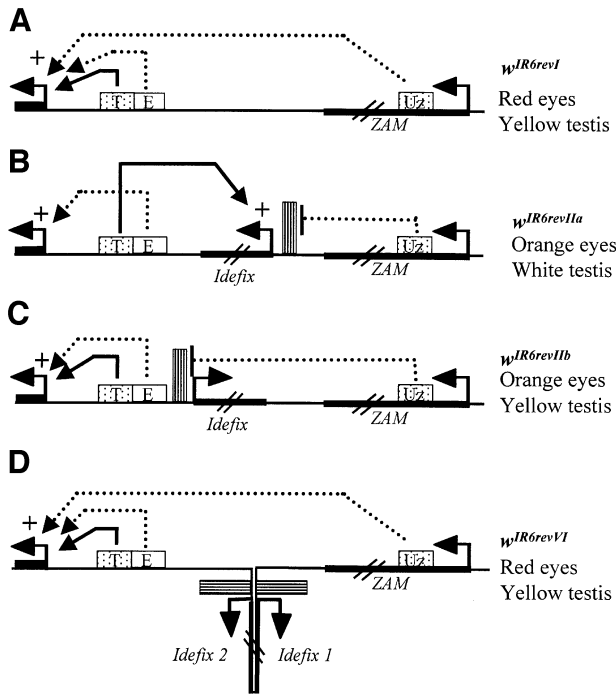


Fig. 5. Models of interactions between ZAM, *Idefix* and *white* in the different *white* alleles. (A), (B), (C) and (D) correspond to the $w^{IR6revI}$, $w^{IR6revIIa}$, $w^{IR6revIIb}$ and $w^{IR6revVI}$ alleles, respectively. The *white* gene is indicated by its promoter, and the beginning of its first exon is shown by a black box. DNA upstream of the gene is shown as a line. The regulatory sequences present in the different *white* alleles studied are represented as follows: enhancers of eyes (E) and testes (T) by boxes; *Idefix* insulator by a hatched rectangle; promoters by arrows; and the 5'-UTR of ZAM bearing an eye enhancer (Uz) by a dotted box. Pointed arrows indicate the activating effects of the eye enhancers. Plain lines indicate the activating effect of the testes enhancer. Names of the alleles and the eyes and testes colors of their corresponding lines are indicated on the right.

(Figure 4B). The five $pLi^{mut}EwW$ lines tested displayed yellow testes, indicating that Li^{mut} does not inhibit interactions between the testes enhancer and the *white* gene. Thus this mutated promoter does not compete with the *white* gene promoter for the shared testes enhancer, although it is transcriptionally active.

These findings show that transcription of competing promoters is necessary but not sufficient for promoter competition. It is the sequence specificity of the core promoters and their inherent properties that form the central machinery mediating competition.

Discussion

The present work describes a new mechanism by which transposable elements can interfere with the host genome in *D. melanogaster*. We have reported elsewhere that the insertions of two retrotransposons, ZAM and *Idefix*, upstream of the transcription start site of *white* bring novel regulatory sequences such as an enhancer and an insulator that modify the proper regulation of *white* in the eyes (Conte *et al.*, 2002; Figure 5). This work reports another type of regulation, namely promoter competition

as a mechanism of transcriptional interference, potentially afforded by such mobile elements. It places transposable elements as regulatory elements able to impose functional restraints on nearby genes.

Idefix promoter competes with the *white* gene promoter for the same enhancer

The present study was initiated after the recovery of novel mutations affecting the eye color of the RevI stock of flies. RevI flies display brick-red eyes due to the presence of a ZAM element 3 kb upstream of w^{IR6} (Leblanc *et al.*, 1999). These new and independent mutations were found to be due to the insertion of an *Idefix* element, 1.7 kb upstream of w^{IR6} between the transcription start site of *white* and the insertion of ZAM. Such insertions had already been reported as responsible to $w^{IR6revII}$ alleles; however, in these new mutational events, *Idefix* was inserted in the opposite orientation, i.e. 3'-5' with regard to the *white* gene transcription instead of 5'-3' in the 12 lines already reported in Desset *et al.* (1999). We denoted the alleles $w^{IR6revIIa}$ when *Idefix* is inserted 5'-3', and $w^{IR6revIIb}$ when it is in the opposite orientation. Lines established were called RevIIa and RevIIb, respectively. Interestingly, all these *Idefix* insertions are responsible for a similar modification of the eye phenotype whatever their orientation, going from brick-red to orange; however, they have a different mutagenic impact on the expression of the downstream *white* gene in testes. Flies bearing $w^{IR6revIIa}$ display white testes while flies bearing the $w^{IR6revIIb}$ allele display yellow testes (Figure 1A and B).

Transgenic assays enabled us to characterize the molecular mechanisms responsible for the mutagenic impact of *Idefix* in testes. We have tested the influence of different fragments of *Idefix* potentially involved in regulation of the $w^{IR6revII}$ alleles and found that when its LTR is present in transgenes designed as follows: '5'LTR3'-enhancers-5'mini-white3'', the *Idefix* LTR prevents the mini-*white* promoter from being activated by its testes enhancer. Transgenic lines bearing this construct that mimics the $w^{IR6revIIa}$ allele display white testes. However, the LTR in this orientation does not block the *white* gene being activated by its eye enhancer also present in the constructs. In the presence or absence of the LTR, no modification of the eye color was observed in any of the transgenic lines obtained.

In RevIIa, as in the transgenic lines, the enhancer-blocking activity of *white* observed in testes cannot be explained by the presence of an insulator sequence within the LTR since insulators block gene expression only when located between a distal enhancer and a target gene. In RevIIa and the tested constructs, the *Idefix* LTR is located upstream of both the enhancer and the target gene (see Figures 2A and 5B). Since the promoter of *Idefix* is present within its LTR, promoter competition established in this tissue offers a better explanation for such a transcriptional interference (as defined by Villemure *et al.*, 2001) that leads to the repression of the downstream *white* gene by the upstream *Idefix* unit. An alternative explanation could be that the LTR possesses a silencer element downstream of the insulator and is responsible for the repressive effect. However, this explanation is very unlikely because the observed transcriptional interference can be prevented

by two point mutations, Li^{mut} and Li^{477} , one of them overlapping with the initiator element (see below).

Transgenic experiments performed with the mutated LTR of *Idefix* (Li^{477}), which suffered a single nucleotide alteration at the promoter start site of *Idefix*, revealed that this inactive promoter is no longer able to interfere in the regulation of the downstream mini-*white* gene in testes. This result shows that the promoter of *Idefix* is engaged directly in the regulation of the $w^{IR6RevIIa}$ allele by competing with the promoter of *white*. Since both *white* and *Idefix* initiate their transcription from a TATA-less promoter, these data indicate that the promoter of *Idefix* displays specific properties allowing its interaction with the testes enhancer and precluding expression of the *white* promoter in this tissue. This specificity can be lost through a single mutation affecting its core promoter such as the Li^{477} mutation that is sufficient to render the interaction inefficient. Additionally, the specific properties of this promoter do not allow the competition to occur in all the tissues where *white* is expressed since it does not take place in the eyes. The reason for this is still unknown; however, it is interesting to note that the promoter of *Idefix* appears to be unable to establish the competition when it is inactive. This observation derives from two main results. First, the promoter present in Li^{477} is unable to direct *Idefix* transcription and it is then not involved in the competition. Secondly, experiments described above indicate that the challenge between the two promoters only occurs in testes where *Idefix* is transcriptionally active and not in the eyes where *Idefix* is inactive (Tcheressiz *et al.*, 2002; Figure 2A).

It must be noted that a mechanism other than promoter competition potentially can explain our experiments: interference of *white* expression resulting from read-through transcription from the upstream *Idefix* promoter. Readthrough transcription may occlude transcription factor-binding sites in a downstream promoter. This mechanism has been suggested previously to explain transcriptional interference at other loci (Proudfoot, 1986; Greger *et al.*, 1998, 2000). Activation of *Idefix* transcription by testis enhancer could therefore result in a reduction of *white* expression in the testes, as is observed. According to a series of data, this mechanism appears unlikely to explain the white color of the $RevIIa$ testes. First, in constructs bearing Li^{mut} , the LTR is able to initiate transcription and thus a potential readthrough of the downstream mini-*white*. Nevertheless, testes of these transgenic lines are yellow as if the mini-*white* gene expression was not occluded by the upstream Li^{mut} transcription. Secondly, in a series of three independent experiments, we searched to detect a potential chimeric transcript initiated in the *Idefix* LTR and ending in the mini-*white* gene. No such transcript was ever detected. Thirdly, in $RevIIa$, which displays white testes, a full-length *Idefix* is inserted upstream of *white*. All signals necessary to start transcription in the 5' LTR and then to stop it within the 3' LTR are present in *Idefix*. Thus, in the $w^{IR6RevIIa}$ alleles, *Idefix* transcription does not pass through the downstream *white* promoter. Nevertheless, testes are white.

All these data stress the fact that promoter competition is the molecular mechanism responsible for $w^{IR6RevII}$ phenotypes in testes.

The specificity of the TATA-less core promoter of *Idefix* is a prime determinant in the competition

As reported above, competition occurs in tissues where both promoters are transcriptionally active; however, we have found that this necessary condition is not sufficient to establish the competition. Transgenic experiments were performed with constructs that display a modification of its promoter so that the sequence was changed but its activity remained. Two main motifs characterized TATA-less promoters: an Inr sequence and a downstream DPE sequence (Kutach and Kadonaga, 2000). They function co-operatively for the binding of TFIID. Both *Idefix* and *white* contain these motifs but they do not conform to the optimal consensus sequence recently reported by Kutach and Kadonaga (2000). Testing an alteration of the *Idefix* TATA-less promoter (Li^{mut}) to create a synthetic promoter conforming to the consensus sequence of Inr core promoters indicated that although Li^{mut} is still active and able to drive *Idefix* expression in testes, it is unable to compete with the *white* gene for its testes enhancer. It is thus clear that the signals necessary for active transcription of a TATA-less promoter such as that created in Li^{mut} are not sufficient to establish competition. The wild-type promoter of *Idefix* appears to be optimal for selective interactions with the *white* enhancer of testes, while a mutated promoter, although able to initiate transcription in testes, is not. The sequence of the active promoter by itself is an additional essential parameter. These findings strengthen the fact that different TATA-less core promoters exist and possess distinct properties that enable them to be activated preferentially by *cis*-regulatory sequences. In that context, it will be interesting in the future to test the strength of promoters and enhancers involved in this transcriptional interference. We suggest that sequences of the Inr and DPE motifs act in concert with factors somehow to restrict the action of an enhancer. The binding of specific proteins to these motifs may be a necessary step in defining the competition.

Competition depends on the orientation of the promoters

The testes phenotypes compared between $RevIIa$ and $RevIIb$ lines reveal that the competition depends on the orientation of the *Idefix* promoter. When the *Idefix* LTR was placed upstream of the *white* enhancers and the mini-*white* gene but in the orientation '3'LTR5'-enhancers-5'mini-*white*3'', it was no longer involved in the competition and thus testes were yellow. In this configuration, the insulator sequence identified in *Idefix* may explain the orientation dependence since it has been identified in the U3 part of the LTR and is thus upstream of the initiation start site of transcription (Conte *et al.*, 2002). In these constructs that mimic $w^{IR6RevIIb}$, the insulator is interposed between the testes enhancer and the promoter of *Idefix*. Therefore, it is able to prevent the enhancer from activating the *Idefix* promoter, as illustrated in Figure 5C. The orientation of promoters involved in competition is certainly an essential determinant for the competition to occur. Several studies have reported that many promoters possess an intrinsic enhancer-blocking activity essential for their proper regulation (O'Donnell *et al.*, 1994; Arkhipova, 1995; Ohtsuki and Levine, 1998). Such an insulator activity coupled with promoters might be a level of control of

promoter competition within the genome. Insulators have been described to protect gene expression from chromosomal position effect by restricting the domain of action of enhancers. These data show that insulators potentially are also required to restrict promoter competition. Recently, Cai *et al.* (2001) have shown that the suHw-mediated blockage of the AE1 enhancer from a downstream promoter depends on the ability of the promoter to compete for AE1. Promoters that are highly competitive for the enhancer are blocked less effectively. In our experiments, the *Idefix* promoter is competitive enough to turn away the testes enhancer from its proper *white* gene. Nevertheless, when interposed, the insulator of *Idefix* is effective to restore the activation of *white* by its enhancer. These results suggest that the ability of a promoter to compete for an enhancer may not be the only parameter keeping the insulator activity through promoter competition.

Two adjacent identical core promoters can block promoter competition

Conditions exist whereby such competition can be suppressed. For example, the presence of two *Idefix* inserted close together and in the opposite orientation, as in the RevIV line, disrupts this promoter competition and leads to flies with yellow testes. RevIV derives from a RevIIa line with the addition of a novel *Idefix* close to the first one but in the opposite orientation (Desset *et al.*, 1999). Two main mechanisms can be proposed to explain this suppressive effect. First, the insulator brought by the second *Idefix* potentially can interfere with the competition since its LTR is positioned in the 3'–5' orientation, as in the *w^{IR6RevIIb}* alleles; its insulator sequence present between the first *Idefix* and the testes enhancer is able to block the action of the enhancer on the *Idefix* promoter. Secondly, it has been shown recently that two adjacent insulators are ineffective in blocking enhancers from a downstream promoter, and we have already reported that this must be why RevIV displays brick-red eyes (Conte *et al.*, 2002). A model for this suppression of insulation is that such insulators may interact with each other through protein complexes bound to them, forming chromatin loop domains that annul their insulator properties (Cai and Shen, 2001; Muravyova *et al.*, 2001). It is tempting to think that such structures might also trap adjacent promoters, preventing them from competing with nearby genes for an enhancer. Further insights into the molecular mechanisms underlying this competition and its suppression by two *Idefix* are necessary to discriminate between these two models. It will be particularly interesting to elucidate whether two *Idefix* insertions (or, in a more general way, two identical sequences) present at two different genomic sites can either allow or restrict transcription of neighboring genes depending on their respective position toward the regulatory sequences of a transcriptional unit.

Concluding remarks

This study brings to light a novel mechanism by which retroelements can interfere with and thus contribute to the regulation of their host. Insertion of mobile elements influences genomic structure and function through several mechanisms that have been widely depicted in the

literature. In addition to disrupting exons, they can insert into non-coding regions and modify gene regulation by adding transcriptional enhancers or insulator sequences delimiting novel autonomous domains of transcription. Our data provide *in vivo* evidence that they can also establish promoter competition with genes located in the vicinity of their insertion and then disrupt endogenous enhancer–promoter communications.

Transcriptional interference involving insulator function and/or promoter competition imprinted by retroelements may disrupt enhancer–promoter communication of neighboring genes and thus contribute to their control in specific tissues and/or developmental stages. It must be stressed that such controls can be found in a line or subgroups of a population, when an insertion recently occurred at a defined locus. However, they can also be established in a whole species if the insertion occurred a long time ago in any ancestor. Identifying vestiges of transposable elements dispersed throughout the genome that have been highly degenerated through evolution but which retained their capacity to initiate transcription or their insulator function should reveal novel *cis*-regulatory elements contributing to eukaryotic gene regulation.

Materials and methods

Plasmid constructs

The regulatory sequences of *white* are located between –1084 and –1465 relative to the transcription start site of the gene (Qian *et al.*, 1992). A 890 bp fragment containing these sequences was amplified by PCR using the primers W1 (ATGCGGATCCGAATTCACGCCTCAGTTCAAGTTAC) and W2 (GTACGGATCCGAATTCACCATTTTCACGGACGAT). The *EcoRI* fragment was inserted in the single *EcoRI* site of pCaSpeR4 (Pirrota, 1988) to give a *P* transformation vector called pEwR. The LTR of *Idefix* amplified by PCR using the primers I1: GTCGACGTGACATATCCATAAG (starting at nucleotide 1 according to the *Idefix* sequence) and I2: CTTCAAGTTGATCAGTACCGTAC (starting at nucleotide 659) was cloned in pGEM-T (Promega). Two different PCR products were obtained, the wild-type LTR and the LTR⁴⁷⁷ (or Li⁴⁷⁷) containing a T→C mutation at nucleotide 477 of *Idefix* (Conte *et al.*, 2002). Also, site-directed mutagenesis was performed as described by Jarrell *et al.* (1988) to introduce an Inr sequence that fits the consensus. The mutated LTR obtained, called Li^{mut}, was identical to the *Idefix* LTR except that its transcription start site ATCAGAG is replaced by TGCAGTT (Figure 3).

The *NotI*–*BamHI* fragments containing the LTR, the Li⁴⁷⁷ or the Li^{mut} were inserted between the FRT in the pKB345 plasmid. The *KpnI* FRT-flanked LTR, Li⁴⁷⁷ and Li^{mut} of *Idefix* fragments were inserted into the *KpnI* sites of pEwR to give, respectively, pLi⁴⁷⁷EwW (Figure 4A) and pLi^{mut}EwW (Figure 4B) transformation vectors.

The *KpnI* FRT-flanked Li^{mut} was inserted into the *KpnI* site of pW6AUGβgal (provided by A. Péliou) to construct the pLi^{mut}LacZ (Figure 3). Staining of testes to measure *lacZ* expression was performed as follows: testes were dissected, fixed with glutaraldehyde and stained with X-gal according to the method of Glaser *et al.* (1986).

P transformation vector

P element transformation vectors containing the *white* reporter gene were introduced into the *Drosophila* germline by injecting *w¹¹¹⁸* embryos as described previously (Rubin and Spradling, 1982). At least five independent transformants were obtained and analyzed for each recombinant *P* element transformation vector.

Fly strains and heat-shock regimes

Fly stocks were maintained on cornmeal–glucose–yeast media at 20°C. The *hsFLP* flies (*w¹¹¹⁸ 70FLP*; *cu kar² Sb/TM6, Ubx e⁺*), kindly provided by Kent Golc, express the *flp* recombinase under the heat shock promoter. Virgin *hsFLP* females were crossed with transgenic males for 24 h on cornmeal–glucose–yeast media at 20°C. Heat shocks of embryos <24 h old were performed as described by Ahmad and Golc (1996).

For each recombinant *P* element transformation vector injected, five independent transgenic lines were heat shocked to compare the eye color of heat-shocked and non-heat-shocked flies.

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